Iron bioavailability from maize and beans: a comparison of human measurements with Caco-2 cell and algorithm predictions[1−4]

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ABSTRACT

Background: An in vitro digestion and Caco-2 cell model may predict iron bioavailability to humans; however, direct comparisons are lacking.

Objective: The objective was to test the differences in iron bioavailability between 2 maize varieties and 2 bean varieties (white beans and colored beans) by comparing human, Caco-2, and algorithm results.

Design: Two randomized, 2×2 factorial experiments compared women's iron absorption from 2 maize varieties (ACR and TZB; $n = 26$) and 2 bean varieties (great northern and pinto; $n = 13$), each fed with and without ascorbic acid (AA) from orange juice. Nonheme iron bioavailability was determined from 2-wk retention of extrinsic radioiron tracers and was compared with Caco-2 cell and algorithm results from identical meals.

Results: Without AA supplementation, women absorbed only about 2% of the iron from the maize or bean meals. The results were unaffected by the variety of either maize or beans. Adding AA (15–20 molar ratios of AA:iron) roughly tripled the iron absorption ($P < 0.0001$) from all test meals. Although the Caco-2 model predicted a slightly improved bioavailability of iron from ACR maize than from TZB maize ($P < 0.05$), it accurately predicted relative iron absorption from the maize meals. However, the Caco-2 model inaccurately predicted both a considerable difference between bean varieties ($P < 0.0001$) and a strong interaction between bean varieties and enhancement by AA ($P < 0.0001$). The algorithm method was more qualitatively than quantitatively useful and requires further development to accurately predict the influence of polyphenols on iron absorption.

Conclusions: Caco-2 predictions confirmed human iron absorption results for maize meals but not for bean meals, and algorithm predictions were only qualitatively predictive. *Am J Clin Nutr* 2007;86:388–96.

KEY WORDS Iron absorption, bioavailability, Caco-2 cells, ascorbic acid, phytic acid, polyphenols, tannins

INTRODUCTION

The prevalence of iron deficiency in resource-poor global regions is exacerbated by a reliance on staple food crops (1–3). Maize, a staple food of several developing nations, has long been regarded as a poor source of bioavailable iron (2, 4–7), and overreliance on maize has contributed to the high rate of iron deficiency in these regions (8, 9). Legumes are considered a rich plant source of iron; however, the bioavailability of this iron from legumes is generally poor (5, 10–12). Biofortification, with an emphasis on the use of crop varieties that have high nutrient bioavailability, has been proposed as a sustainable strategy for reducing iron deficiency (13, 14).

Before expensive, on-site efficacy trials to assess the effect of biofortification on subjects' nutritional status, human bioavailability measurements should be conducted (15). It would be advantageous if human bioavailability testing could be based on preliminary in vitro screening methods to efficiently identify promising plant cultivars. An in vitro digestion and Caco-2 cell model (16), which simulates digestion and intestinal uptake, uses a dialysis membrane to protect cells from hydrolytic enzymes and measures cellular ferritin formation as an indicator of iron uptake. Indirect comparisons with human data indicate that the Caco-2 model can predict the influence of ascorbic acid (AA) and polyphenolic compounds on relative iron bioavailability (17). However, this model has not been directly validated by simultaneously testing identical meals in both humans and the in vitro system.

Oikeh et al (1) used the in vitro digestion and Caco-2 cell model to screen 20 early-maturing tropical maize varieties. Compared with TZB-SR (TZB), a control variety widely grown in the savannas of Nigeria, ACR90POOL16-DT (ACR), a variety with improved resistance to diseases, high yield potential, and tolerance to drought, had a similar content of iron that was 25–46% more bioavailable, according to the in vitro model. Thus, ACR was recommended for human testing (1).

It has been proposed that iron-binding polyphenolic compounds in the hulls may reduce iron bioavailability from colored

beans but not from white beans (18). Results from the in vitro Caco-2 cell model suggested substantially less iron bioavailability from colored beans than from white beans (19) and indicated that AA enhanced iron bioavailability from white beans but not from colored beans. Further investigation was warranted, because colored beans are a staple food in regions with widespread iron deficiency (18).

The purpose of the current investigation was to compare human iron absorption between 2 maize varieties (TZB and ACR) and between 2 bean varieties (the pinto, which is a mottled red color, and the great northern, which is white) with and without the addition of AA, and to directly compare the results from the in vitro Caco-2 cell model and the algorithm predictions with the human iron bioavailability results.

SUBJECTS AND METHODS

General design and power testing

Iron bioavailability from maize and beans was separately evaluated in 2 human experiments. Each experiment was designed as a 2 × 2 factorial to compare the effects of crop variety (ACR compared with TZB maize or colored pinto beans compared with white great northern beans) and of AA from orange juice on iron absorption. Within each experiment, women consumed each food variety twice, once with and once without orange juice. During each 29-d experiment, the participants resided in their own homes. On days 1, 2, 15, and 16, the participants came to the testing center after having fasted overnight, where they consumed breakfast meals radiolabeled extrinsically for nonheme iron, a commonly accepted labeling technique that has been specifically tested with maize and beans (5). Iron absorption was assessed from red blood cell radioisotope incorporation and whole-body scintillation counting. The same meals were tested by using an in vitro digestion and Caco-2 cell model and were evaluated by an algorithm for comparison with the human results.

The maize study was planned on the basis of published in vitro Caco-2 analyses (1), which indicated that ACR had 25–46% more bioavailable iron than did TZB. Adequate statistical power was achieved to detect this relatively small difference by evaluating differences in maize varieties both with and without AA, thereby requiring fewer total subjects. Accordingly, 26 women were studied, which provided 90% statistical power (α level of 0.05) to detect a 33% difference or 70% power to detect a 25% difference between maize varieties. In addition, the use of a modest amount of AA (39 mg) in the maize experiment enabled a direct comparison of the enhancement by AA in vitro and in vivo and a demonstration of the relative effect of crop variety compared with ascorbate-containing foods on iron absorption. For power calculations, it was estimated that this amount of AA would increase iron absorption by at least 40%, with greater enhancement depending on the phytic acid content of the meal (20).

On the basis of the in vitro analyses of the same bean meals, the bean study with humans was powered to detect an interaction between bean color and AA, in which iron was twice as bioavailable from white beans as from colored beans and was further doubled in white beans, but not in colored beans, by serving AA with the meal. Thirteen subjects provided 90% power to detect this interaction (α level of 0.05). For power calculations, it was

TABLE 1
Characteristics of the participants in the maize and bean experiments

	Maize ($n = 26$)	Beans ($n = 13$)
Age (y)	33 ± 9[1]	33 ± 9
Weight (kg)	64 ± 9	64 ± 8
BMI (kg/m^2)	24 ± 2	24 ± 2
Ferritin (μg/L)[2]	28 (3–248)	31 (6–93)
Hemoglobin (g/L)	13 ± 0.6	14 ± 0.6

[1] $\bar{x} \pm$ SD (all such values).
[2] Geometric $\bar{x}$; range in parentheses.

estimated that 100 mg AA would at least double iron absorption, irrespective of the phytic acid content of the meal (20). More AA was used in the bean study than in the maize study (100 compared with 39 mg) to enable confirmation of the significant interaction between AA and bean variety as detected with the Caco-2 system.

Participants

Women were recruited for the present study through public advertisements. Participants (**Table 1**) in the maize ($n = 26$) and bean ($n = 13$) experiments were verified as being in generally good health from the results of total blood cell counts, liver and kidney function tests, urinalyses, and blood pressure measurements. No subject participated in both experiments. Reasons for exclusion from the present study included a hemoglobin concentration <12 g/L, the routine use of medications other than oral contraceptives or hormone replacement therapy, or body mass index (in kg/m^2) <18 or >30. Participants had neither been pregnant nor had lactated within the previous 6 mo. Subjects discontinued the use of all highly iron-fortified products, vitamins, minerals, or herbal supplements and refrained from donating blood and plasma for the duration of the study. All procedures and potential risks were explained, and all participants gave written informed consent. The present study received approval for human subjects by the University of North Dakota Institutional Review Board and its Radioactive Drug Research Committee and by the US Department of Agriculture's Radiological Safety Committee.

Meals

The control variety of maize, TZB, was selected because it had been widely grown across Nigeria since 1986 (1). The test maize, ACR, was selected because its iron content was similar to that of TZB but was more bioavailable, as shown in a previous in vitro Caco-2 cell analysis (1).

Maize grown at the International Institute of Tropical Agriculture station in Ibadan, Nigeria, under normal growing conditions was hand harvested, dried, and shipped to the US Department of Agriculture–Agricultural Research Service facility (Grand Forks, ND) for the human study. The kernels were rinsed with cold running water to remove debris and were allowed to air dry overnight. Maize was ground into a semifine powder with an electronic, die-cast aluminum food grinder (Grain Mill, model GMA; KitchenAid, St Joseph, MI) and was refrigerated in an airtight container until it was cooked. Meals were prepared in batches by using a ratio of 50 g ground maize to 300 mL water per serving. Water was brought to a boil in a glass pot, and the ground maize was stirred in. The uncovered mixture simmered for 15

min with occasional stirring and was then cooled at room temperature. Once cool, individual portions were placed in plastic containers with 15 g sucrose and were frozen for later use. Each variety of maize was served twice, once with and once without 80 mL orange juice, which provided ≈39 mg AA. Orange juice, reconstituted from frozen concentrate (Minute Maid Premium Pulp Free; Coca-Cola Company, Houston, TX), was immediately covered, refrigerated, and consumed within 2 h of preparation.

Pinto and great northern beans were grown under normal cultivation conditions in North Dakota or Minnesota (Northarvest Bean Growers Association, Frazee, MN). After being rinsed with cold water, 454 g dried beans with 1835 mL water, 0.8 g garlic powder, and 8 g onion powder were cooked in a slow-cooker on high heat for 4.5 h. Salt (7 g) was added, and the beans were cooked for another 30 min. Individual portions containing 100 g cooked beans plus 50 g cooking liquid were frozen. Each bean variety was served twice, once with and once without 230 mL reconstituted orange juice, which provided ≈112 mg AA.

Food analyses

Meal samples were digested with concentrated nitric acid and 70% perchloric acid according to method (II)A of the Analytic Methods Committee (21). Digestates were analyzed for iron, calcium, and zinc contents by using inductively coupled argon plasma emission spectrophotometry. Phytate was extracted from lyophilized meal samples by using 2.4% HCl and was separated by using an anion-exchange column according to method 986.11 of the Association of Official Analytical Chemists (22). Extracts were digested with sulfuric and nitric acids to liberate phosphorus, which was quantified by using ultraviolet spectrophotometry. Phytate was quantified by assuming 6 mol phosphorus per mole phytic acid. AA in reconstituted orange juice was oxidized to dehydroascorbic acid and quantified by fluorescence according to method 967.22 of the Association of Official Analytical Chemists (23). To test the stability of AA in prepared orange juice, refrigerated samples were tested at 0, 1, and 2 h after preparation. The AA concentration of the prepared juice was 0.5 ± 0.02 mg/mL ($\bar{x} \pm$ SEM: 2.8 ± 0.1 mmol/L) and remained stable for 2 h. Tannin equivalents, or iron-binding phenolic (galloyl) groups, were measured spectrophotometrically after extraction with dimethylformamide and the addition of ferric ammonium sulfate (24).

Iron absorption measurements

Within each experiment, the 4 randomly assigned meals were served as 2 sets on 2 consecutive days separated by 2 wk (days 1, 2, 15, and 16). The first meal of each set (days 1 and 15) was extrinsically labeled with 1 μCi ^{59}FeCl$_2$ in <0.2 μg elemental Fe, and the second meal of each set (days 2 and 16) was extrinsically labeled with 3 μCi ^{55}FeCl$_2$ in <0.03 μg elemental Fe. Meals were served after the participants had fasted overnight, and the participants refrained from consuming anything by mouth, other than water, for 4 h after completing the meals.

Two-week isotope retention, assessed with whole-body scintillation counting and red blood cell radioisotope incorporation measurements, was used to determine iron absorption. A whole-body count representing 100% of the administered dose of ^{59}Fe was obtained 2–5 h after the meals were consumed (days 1 and 15). For meals labeled with ^{59}Fe, iron absorption was calculated

as the percentage of whole-body radioactivity that remained after 2 wk (day 14 for the first 2 meals and day 29 for the last 2 meals), corrected for physical decay and background radioactivity measured 1 d before the meals were consumed. Concentrations of ^{55}Fe and ^{59}Fe in blood were measured on days 15 and 29 (25). For meals labeled with ^{55}Fe (not a γ-emitting isotope and not detectable by whole-body scintillation counting), iron absorption was calculated by assuming that a similar fraction of each absorbed isotope was incorporated into blood, an assumption that has been confirmed in animals (26). Thus, whole-body ^{55}Fe fractional retention was equal to whole-body ^{59}Fe fractional retention multiplied by the ratio of ^{55}Fe to ^{59}Fe fractions incorporated into the blood 2 wk later, with corrections for background and radiological decay. This dual-isotope, whole-body counting method eliminated the need to estimate total blood volume or to assume 80% erythrocyte incorporation of the absorbed radioisotope, and it decreased the duration of the volunteer involvement by half. Results from a previous study (27) indicated that iron excretion was minimal during the 4 wk after radioisotope administration. Thus, there was no need to correct for endogenous excretion of absorbed radioisotope.

Additional blood analyses

Fasting venous blood (30 mL) drawn on days 1, 15, and 29 was analyzed for hemoglobin by using a Celldyne 3500 System (Abbott Laboratories, Abbott Park, IL). Serum ferritin was measured by immunometric assay (Diagnostic Products Corporation, Los Angeles, CA) with the use of beads coated with murine monoclonal anti-ferritin antibodies. C-reactive protein was measured by nephelometry (Behring Diagnostics Inc, Westwood, MA) and was used as an indicator of inflammation. No ferritin values were eliminated on the basis of the C-reactive protein measurements.

In vitro iron bioavailability

Frozen, prepared maize and bean meal samples were shipped to the US Department of Agriculture–Agricultural Research Service, Ithaca, NY, for assessment of iron bioavailability with the in vitro digestion and Caco-2 cell culture model (16). For logistic reasons, the maize samples were tested concurrently in vitro and in vivo. Commercial orange juice for the test meal was purchased separately at the sites of the in vitro and in vivo testing. The in vitro testing used the same volumetric ratio of orange juice to food, and because the AA concentration of the orange juice used in vitro was 48% lower than that tested in vivo, additional AA was added to match the in vivo concentration (0.5 mg/mL or 2.8 mmol/L). After digestion, 2-g samples of meal digests were added to Caco-2 cells, and cellular ferritin formation was measured by radioimmunoassay (FER-Iron II Ferritin Assay; RAMCO Laboratories, Houston, TX). Caco-2 cell protein content was measured with a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Baseline ferritin concentrations were obtained with Caco-2 cells that were not exposed to digested food samples.

Statistical analyses

The analyzed composition of the food varieties was compared by using Student's *t* test (SAS version 9.1; SAS Institute Inc, Cary, NC). Serum ferritin and iron absorption measurements were logarithmically transformed for statistical analyses and are

presented as geometric means (+1 SEM, −1 SEM). Human iron absorption measurements are reported as observed and were used to calculate absorption ratios between meals. To facilitate comparisons with other studies, iron absorption was also normalized to a serum ferritin concentration of 23 μg/L, which is the iron status that corresponds with 40% absorption of a reference dose (20). Normalized values for each subject were calculated as follows:

$$\ln(\text{Normalized \% iron absorption}) = \ln(\text{\% iron absorbed})$$
$$+ \ln(\text{ferritin; in } \mu\text{g/L}) - \ln(23 \ \mu\text{g/L}) \quad (1)$$

The effects of crop variety and AA on human iron absorption were compared by using repeated-measures analysis of variance (ANOVA) with Tukey's post hoc contrasts to detect significant differences between pairs of means. The effects of crop variety and AA on Caco-2 cell ferritin formation were compared by using 2-factor ANOVA with Tukey's post hoc contrasts.

To enable additional comparisons between methods, data were expressed as absorption ratios relative to the results for one treatment (TZB and great northern beans for the maize and bean experiments, respectively). Human absorption ratios were calculated for each participant according to micrograms of iron absorbed, accounting for differences in both the percentage absorption and the food iron content. Caco-2 cell absorption ratios based on cell ferritin formation were modified to predict human absorption ratios by using the formula from Yun et al (17):

$$\ln(\text{Human absorption ratio}) = 0.6401$$
$$\times \ln(\text{Caco-2 absorption ratio}) \quad (2)$$

The Caco-2 absorption ratio in Equation 2 does not adjust for baseline ferritin values when the cells are tested without food. Although baseline ferritin concentrations were undetectable in the present study, in most studies with this model, baseline ferritin is detectable at values of ≈2–3 ng ferritin/mg cell protein. Relative absorption ratios observed in the women were compared with those predicted with Caco-2 cells by using Student's t tests.

Significant differences for all analyses were noted at $P < 0.05$ (2-tailed analyses). For reference, results were also compared with the predictions from the algorithm of Hallberg and Hulthen (20) by using the analyzed meal AA, phytic acid phosphorus (phytate-P), and tannic acid (TA) equivalent contents with the following formula:

$$\text{\% Absorption} = 22.1 \times f_1 \times f_2 \times f_3 \quad (3)$$

where

$$f_1 = 1 + 0.01 \times \text{AA} + \log(\text{phytate-P} + 1) \times 0.01$$
$$\times 10^{0.8875 \times \log(\text{AA} + 1)} \quad (4)$$

$$f_2 = 10^{-0.30 \times \log(\text{phytate-P} + 1)} \quad (5)$$

and

$$f_3 = (1 + 0.01 \times \text{meat})$$
$$\times 10^{0.4515 - [0.715 - 0.1825 \times \log(1 + \text{AA})] \times \log(1 + \text{TA})} \quad (6)$$

The algorithm in Equation 3 uses base-10 logarithms; meat is expressed in grams (zero for the present meals), and AA,

TABLE 2
Analyzed nutrient composition of the maize test meals

	TZB maize	ACR maize	P^1
Iron (mg/meal)	0.71 ± 0.01^2	0.84 ± 0.02	<0.0001
Calcium (mg/meal)	16 ± 1.6	17 ± 1.1	0.56
Zinc (mg/meal)	1.3 ± 0.1	1.7 ± 0.1	<0.01
Phytate (mg/meal)	418 ± 6.2	446 ± 5.8	<0.001
Tannic acid equivalents (mg/meal)	6.4 ± 1.3	5.7 ± 1.1	0.52
For meals with added orange juice			
Ascorbic acid (mg/meal)	39 ± 3	39 ± 3	—
Ascorbic acid:Fe (molar ratio)	17	15	—

[1] Student's t test. Statistical comparisons were not conducted with ascorbic acid, which was determined from the same orange juice added to both maize varieties tested.
[2] $\bar{x} \pm$ SD (all such values); $n = 3$.

phytate-P, and TA are expressed in milligrams per meal. Factors f_1, f_2, and f_3 of this algorithm adjust for AA, phytic acid, and TA equivalents, respectively, and include interactions between these components. The multiplier that adjusts for TA (f_3) is not allowed to exceed one. Because of the difficulties in measuring TA content in the beans, which is discussed in Results, the algorithm calculations of iron absorption from beans were applied both with and without adjustments for TA (f_3).

RESULTS

On the basis of the meal analyses (**Tables 2** and **3**), the ACR maize variety had nearly 20% more iron than did the TZB variety ($P < 0.0001$). ACR also contained ≈25% more zinc ($P < 0.01$) and 7% more phytate ($P < 0.001$) than did TZB. The iron content of the pinto bean was not significantly different from that of the great northern bean. The pinto bean had 25% less calcium ($P < 0.0001$) and 7% less phytate ($P < 0.001$) than did the great northern bean.

Fractional iron absorption from both maize and bean meals without AA was low: ≈2% (**Tables 4** and **5**). For both maize and beans, the crop varieties did not differ significantly in either the percentage or the total amount of iron absorbed by

TABLE 3
Analyzed nutrient composition of the bean test meals

	Great northern bean	Pinto bean	P^1
Iron (mg/meal)	1.9 ± 0.2^2	2.1 ± 0.2	<0.20
Calcium (mg/meal)	78 ± 3.1	58 ± 2.3	<0.0001
Zinc (mg/meal)	1.1 ± 0.1	1.2 ± 0.1	0.25
Phytate (mg/meal)	289 ± 0.3	270 ± 1.7	<0.001
For meals with added orange juice			
Ascorbic acid (mg/meal)	112 ± 8	112 ± 8	—
Ascorbic acid:Fe (molar ratio)	19	17	—

[1] Student's t test. Statistical comparisons were not conducted with ascorbic acid, which was determined from the same orange juice added to both bean varieties.
[2] $\bar{x} \pm$ SD (all such values); $n = 3$.

TABLE 4
In vivo and in vitro assessments of iron bioavailability from maize meals with or without orange juice[1]

	TZB	TZB + AA	ACR	ACR + AA	Variety	Ascorbate	V × A
						P^2	
Women's iron absorption							
Observed							
(%)	2.0 (1.6, 2.5)[3]	7.3 (5.8, 9.1)	2.2 (1.7, 2.7)	7.0 (5.6, 8.7)	NS	0.0001	NS
(μg/meal)	14 (11, 18)	51 (41, 64)	18 (15, 23)	58 (46, 73)	NS	0.0001	NS
Normalized (%)[4]	2.4 (2.1, 2.9)	8.8 (7.4, 10.4)	2.6 (2.2, 3.1)	8.4 (7.1, 10.0)	NS	0.0001	NS
Caco-2 cell ferritin (ng/mg protein)[5]	5.7 ± 0.5	41.7 ± 1.3	7.6 ± 1.7	45.8 ± 1.5	0.04	0.0001	NS
Algorithm with tannins (% absorption)[6]	4.3	10.2	4.6	10.1	—	—	—

[1] AA, ascorbic acid.

[2] Main effects of an analysis of variance. Statistical variation is not provided for the modified algorithm predictions because there were no repeated measures. V × A, interaction between maize variety and AA.

[3] Geometric $\bar{x}$; −1 SEM and +1 SEM values in parentheses (all such values); $n = 26$.

[4] Absorption was normalized to a serum ferritin concentration of 23 μg/L (*see* text).

[5] Arithmetic $\bar{x}$ ± SEM; $n = 6$.

[6] Results from the application of the algorithm from Hallberg and Hulthen (20), for comparison with the women's normalized results.

the women. However, because of the difference in iron content, total iron absorption tended ($P = 0.06$) to be slightly greater (4–7 μg/meal) from the ACR than from the TZB maize (Table 4). Adding AA to meals significantly enhanced iron absorption, from ≈2% to 7% for maize ($P < 0.0001$) and from 2% to 6% for beans ($P < 0.0001$; Tables 4 and 5). Iron absorption was inversely related to serum ferritin for all 4 maize meals ($n = 26$) and for 3 of 4 bean meals ($n = 13$; data not shown); the one correlation that was not significant became significant when one outlying value was excluded. This confirms that women with low body iron stores absorb iron more efficiently and benefit from greater differences in the absolute amounts of iron absorbed because of the additional AA. However, the relative iron absorption (between meals, expressed as a ratio) was not significantly affected by iron status (data not shown), which is consistent with the concept that the relative absorption results between different food combinations are unaffected by body iron stores. To facilitate comparisons with the algorithm calculations and with other studies, the absorption data were also listed when normalized

to a serum ferritin concentration of 23 μg/L, which is the iron status that corresponds with 40% absorption of a reference dose (20; Tables 4 and 5).

Without AA, Caco-2 cell ferritin formation from maize meals and great northern bean meals but not from pinto bean meals significantly exceeded a baseline cell ferritin of 0 ± 0 ng/mg protein without food. A zero ferritin response with the pinto bean meal was in accordance with the results from previous Caco-2 cell studies, in which it was necessary to add AA to maize and rice samples to detect cellular ferritin formation consistently greater than background (1, 28).

Although the Caco-2 cell model detected slightly more bioavailable iron from ACR than from TZB maize (on the basis of the main effect of the ANOVA, $P < 0.05$; Table 4), this difference was not substantial enough to be significant in separate comparisons of the ACR and TZB mean results, either with or without orange juice (Table 4; Tukey's contrasts). Caco-2 cell ferritin formation increased significantly from both maize meals with the addition of AA (Table 4).

TABLE 5
In vivo and in vitro assessments of iron bioavailability from bean meals with or without orange juice[1]

	Great northern	Great northern + AA	Pinto	Pinto + AA	Variety	Ascorbate	V × A
						P^2	
Women's iron absorption							
Observed							
(%)	2.2 (1.7, 3.0)[3]	6.6 (4.9, 8.9)	1.6 (1.2, 2.1)	5.4 (4.0, 7.3)	NS	0.0001	NS
(μg/meal)	42 (31, 56)	124 (92, 166)	33 (25, 45)	116 (86, 155)	NS	0.0001	NS
Normalized (%)[4]	3.0 (2.3, 4.0)	9.0 (6.8, 11.9)	2.1 (1.6, 2.8)	7.4 (5.6, 9.7)	NS	0.0001	NS
Caco-2 cell ferritin (ng/mg protein)[5]	53.4 ± 2.5[a]	159.1 ± 4.9[b]	0 ± 0[c]	6.0 ± 0.2[d]	0.0001	0.0001	0.0001
Algorithm without tannins (% absorption)[6]	6.0	20.2	6.1	20.2	—	—	—

[1] AA, ascorbic acid. Values in the same row with different superscript letters are significantly different, $P < 0.05$.

[2] Main effects of an analysis of variance. Statistical variation is not provided for the modified algorithm predictions because there were no repeated measures. V × A, interaction between bean variety and AA.

[3] Geometric $\bar{x}$; −1 SEM and +1 SEM values in parentheses (all such values); $n = 13$.

[4] Absorption was normalized to a serum ferritin concentration of 23 μg/L (*see* text).

[5] Arithmetic $\bar{x}$ ± SEM; $n = 6$.

[6] Modified from Hallberg and Hulthen (20), adjusted only for meal AA and phytic acid contents but not for polyphenol or tannin content (*see* Subjects and Methods and Results), for comparison with the women's normalized results.
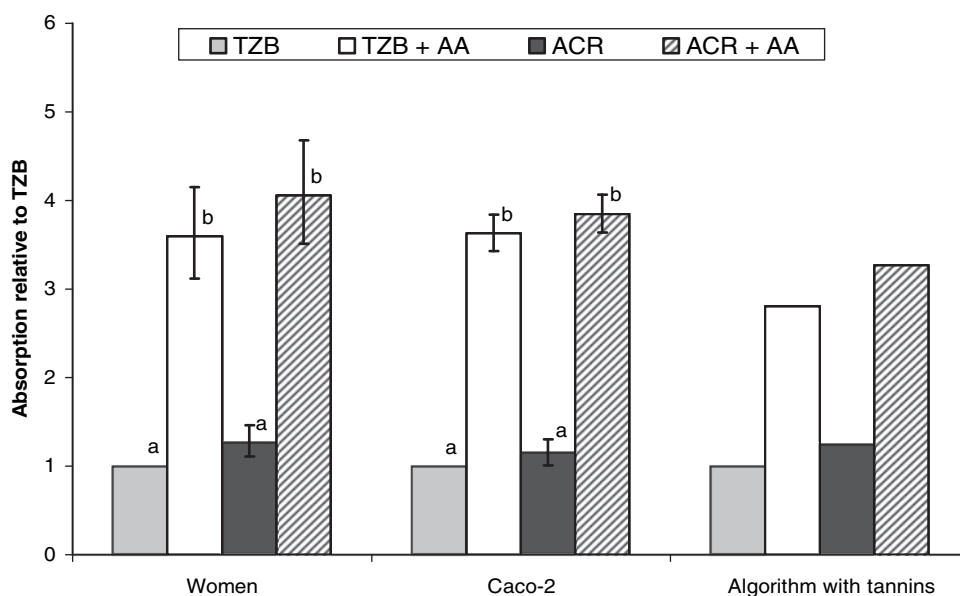
FIGURE 1. Mean (±SE) iron absorption from maize meals. Relative absorption ratios (TZB = 1) were calculated separately for each method, which enabled comparisons independent of subjects' iron status. For comparison with the human results, absorption ratios from Caco-2 cell data were modified by using the formula of Yun et al (17). Women's relative iron absorption from maize meals was better predicted in vitro by the Caco-2 cell model than by the algorithm. These relative ratios are based on the absolute data summarized in Table 4. AA, ascorbic acid. Within a method, different letters indicate significant differences by Tukey's post hoc comparisons, $P < 0.05$. Between human and Caco-2 cell methods, there were no significant differences in relative absorption. Statistical tests were not conducted with the algorithm predictions because there were no repeated measures.

Without the addition of AA, the great northern bean variety induced significantly more cellular ferritin formation than did the pinto bean, which had nondetectable results (159 ± 5 and 0 ± 0 ng/mg protein, respectively, $P < 0.0001$; Table 5). Orange juice significantly enhanced cellular ferritin formation with great northern beans (from 53 to 159 ng/mg protein, $P < 0.0001$) and slightly increased cellular ferritin formation with pinto beans (from 0 to 6 ng/mg protein, $P < 0.0001$). The stronger influence of AA on ferritin formation with great northern beans than with pinto beans resulted in a significant bean variety × AA interaction effect ($P < 0.0001$).

To enable further methods comparisons, relative absorption or absorption ratios were calculated, including the application of the conversion factor formulated by Yun et al (17) for the Caco-2 cell results. Relative iron absorption was not significantly different between Caco-2 cell and human results for maize (**Figure 1**). However, women's relative iron absorption from beans was poorly predicted by the Caco-2 cell method, with significantly different results for relative absorption for all 3 absorption ratio comparisons (**Figure 2**).

The results were further compared with iron absorption predictions from a mathematical algorithm on the basis of the effects of AA, phytic acid, and polyphenols (as TA equivalents; 20). These predictions are for subjects whose serum ferritin concentration was 23 μg/L (20). Compared with the women's normalized iron absorption, the algorithm somewhat overestimated the percentage of absorption for maize (Table 4). Expressed as relative absorption values, the algorithm quantitatively underestimated the enhancing effect of AA for the maize meals (Figure 1).

We encountered unexpected difficulties when we attempted to measure TA equivalents and apply the Hallberg and Hulthen algorithm (20) to the bean meals. Using the same method (24) as was used by Hallberg and Hulthen (20), we confirmed the finding of Hallberg and Hulthen (20) of similar TA equivalents in white

(great northern) and red (pinto) beans. However, our somewhat greater results are not presented here because the analytic recovery of added TA was consistently incomplete (<50%) with beans, in contrast with the complete recovery (100–104%) with maize. We concluded that this method for determining iron-binding polyphenols could not be applied to beans. Without the TA adjustment, the algorithm overestimated normalized absorption from the bean meals (Table 5) but produced a pattern of relative absorption similar to the women's results (Figure 2). The inclusion of the TA adjustment would likely weaken these algorithm results for beans because the measured tannins would lower the prediction of percentage absorption only for those bean meals without added AA and thereby overestimate the relative enhancing effect of AA.

DISCUSSION

Maize, a staple food crop of many underdeveloped countries, is a poor source of bioavailable iron (2, 4–7). Oikeh et al (1) recommended human testing of the TZB and ACR maize varieties because of the findings of similar iron content but higher in vitro (Caco-2 cell) iron bioavailability from ACR than from TZB. In this follow-up investigation, ACR had a slightly greater iron content. The resulting ≈25% difference in total iron absorption between maize varieties was similar in magnitude for both human and Caco-2 cell results, although the difference was not significant with the former and was only marginally significant with the latter. Whereas the women's total iron absorption from ACR tended to be higher than from TZB because of the higher iron content of ACR, the difference of ≈4 μg per meal was small compared with the iron amounts used in effective fortification trials (29–32) and would likely provide no measurable benefit for consumers in developing regions.
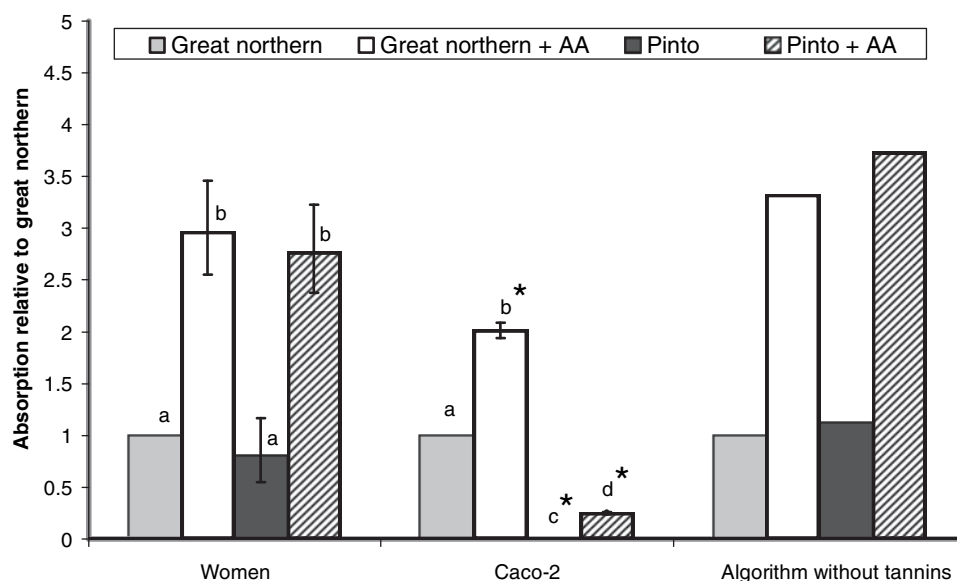
FIGURE 2. Mean (±SE) iron absorption from bean meals. Relative absorption ratios (great northern = 1) were calculated separately for each method, which enabled comparisons independent of subjects' iron status. For comparison with the human results, absorption ratios from Caco-2 cell data were modified by using the formula of Yun et al (17). Women's relative iron absorption from bean meals was poorly predicted in vitro by the Caco-2 cell model. The algorithm predictions are presented without adjustment for tannins because they could not be quantified (*see* text); with the algorithm (20), additional tannins would have reduced iron absorption mainly from the meals without ascorbic acid (AA), thereby making the relative absorption prediction less accurate. These relative ratios are based on the absolute data summarized in Table 5. Within a method, different letters indicate significant differences by Tukey's post hoc comparisons, $P < 0.05$. Between methods (human compared with Caco-2 cell methods), an asterisk indicates significant differences by Student's *t* test, $P < 0.05$. Statistical tests were not conducted with the algorithm predictions because there were no repeated measures.

Although the small Caco-2 difference between maize varieties (Table 4) shown in the present study is consistent with the results of Oikeh et al (1), in retrospect, the importance of this small difference is questionable. Oikeh et al (1) took care to foster the reproducibility of the Caco-2 results: 5 replicates were tested against the TZB reference control for each maize variety at 3 locations, and the sampling and testing were repeated (1). However, the original bioavailability differences were slight, with ACR iron bioavailability being only 46% ($n = 15$, $P < 0.05$) and 25% ($n = 15$, NS) greater than that of TZB and with improved bioavailability of ACR in just 2 of 3 locations for which data were provided. With hindsight, this difference was insufficient to justify additional testing. These results emphasize the need, before human bioavailability tests are done, to select crop varieties with more consistent and substantial differences in iron content and in vitro bioavailability that are highly reproducible across seasons, locations, and growing conditions.

Although beans are a richer source of iron than is maize, the women's fractional iron absorption from beans was also low, with no apparent difference between varieties. These results are similar to previous human studies that showed poor iron absorption from beans when varieties of different colors were tested separately (5, 10–12).

The Caco-2 cell model inaccurately predicted lower iron bioavailability from pinto beans than from great northern beans and a lesser enhancing effect of AA with pinto beans than with great northern beans. The algorithm predictions (20) were also inaccurate unless the polyphenol or tannin adjustment was eliminated. Although previous in vitro analyses (18, 33, 34) suggested that polyphenolic compounds, which contribute pigment to colored beans, bind iron and render it unavailable for absorption,

this has not been confirmed in animals (35, 36), nor was it confirmed in the present human study. Bean pigmentation is commonly attributed to flavonoid, not tannin, polyphenols. It was beyond the scope of the present study to determine the specific pigments accountable for the Caco-2 differences, especially because these differences did not occur in humans. Polyphenol analysis methods vary considerably, with different results. With the Brune et al (24) method (which had poor analytic recovery with beans in the present investigation), the difference in tannin content between pinto and great northern beans was reported as minimal (20), compared with differences as small as 30% and as great as 300% with the use of other analytic methods (37). The application of the more extreme of these differences to the algorithm would tend to overestimate the effect of bean variety and its interaction with AA similarly to the Caco-2 cell results. It may also be relevant that AA enhancement of iron absorption in the presence of polyphenols such as TA, which was observed in humans (38–41), was not observed in the Caco-2 cell system (42). Further research is needed to develop a fully validated analytic method for measuring the polyphenols that influence human iron absorption. Although tannins from coffee and tea have clearly been shown to reduce iron bioavailability (20), polyphenol measurements of mixed diets have not been shown to be predictive (43).

Further development of algorithm and Caco-2 cell methods to correspond with the human results is needed to facilitate efficient and economic screening of foods for possible biofortification. This Caco-2 system is limited to measuring the entrance of iron from a food digestate into the mucosal cell, whereas iron bioavailability for humans also involves serosal transfer from the mucosal cell and subsequent transport and utilization. Additional

testing of in vitro conditions is warranted, because cell passage and differentiation, incubation medium, time, pH, method of cell protection from digestive enzymes, and use of radioiron variables compared with Caco-2 cell ferritin response variables may all influence in vitro results. The algorithm predictions were more qualitatively than quantitatively useful for predicting the present human absorption results.

Iron absorption from these high-phytate, vegetarian meals was significantly enhanced by the addition of AA, which confirms previous work (20, 44–46) and shows the benefit of incorporating AA-rich foods into diets based on staple food crops. Although research on the effectiveness of AA fortification for improving the iron status of developing populations has been inconsistent (47, 48), AA in the same meal consistently enhanced food iron absorption (20, 44–46). Unfortunately, low intakes of AA are common in resource-poor regions (49), and considerable intakes of AA would be required to enhance iron absorption from diets based on these high-phytate foods.

In addition to evaluating the bioavailability results from the Caco-2 model, short-term human absorption results such as these from US women can serve as a preliminary basis for biofortification decisions in plant breeding programs and human efficacy trials in targeted regions. Although the efficiency of iron absorption correlates inversely with body iron status, relative bioavailability measurements appear to be independent of iron status (27, 50–52). This was confirmed in the present study (data not shown) and has been observed in developing countries in subjects with much lower iron stores (51, 52), including 870 rural Venezuelans, one-third of whom had serum ferritin concentrations <12 μg/L. Thus, the relative iron bioavailability between foods can be accurately evaluated by using subjects with a range of iron stores, yielding preliminary results that are applicable to populations with poor iron status before more costly human efficacy trials within target regions.

Although the present results are based on single meals, prior equilibration to the meals in the present study would be unlikely to influence the results for subjects with low iron stores. Partial adaptation in iron absorption, which reduced the difference between low and high bioavailability diets from 8- to 4-fold after 10 wk in US men with adequate iron status (53), was minimal in US women (54). Whereas on-site efficacy trials will ultimately be needed to confirm biofortification plans, preliminary short-term human absorption comparisons such as in the present study are useful for detecting differences in the bioavailability of food iron.

In conclusion, iron bioavailability did not differ between maize or bean varieties. Although women tended to absorb slightly more iron from ACR than from TZB, ACR provided little benefit over TZB maize. Fractional iron absorption from both pinto and great northern beans was low and, in contrast with Caco-2 in vitro results, was not influenced by bean color. AA consistently enhanced iron absorption from all these meals. The algorithm method was more qualitatively than quantitatively useful and requires further development to accurately predict the influence of polyphenols on human iron absorption. The Caco-2 cell in vitro system accurately predicted relative iron absorption from maize meals but inaccurately predicted differences in bean varieties and the interaction of bean variety with AA.

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